(12) UK Patent Application (19) GB (11) 2 334 578 (13) A

(43) Date of A Publication 25.08.1999

(21) Application No 9803447.3

(22) Date of Filing 18.02.1998

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(51) INT CL[6]
C12Q 1/48 , G01N 33/573 33/574 // G01N 33/68

(52) UK CL (Edition Q)
G1B BAB BAD B108 B121 B300 B309 B728
U1S S1303 S1333 S1337 S2419

(56) Documents Cited
WO 97/04316 A1
Medecine Science 1996,12(2),165-173 Leukemia 1995,9,1382-1388 Int.J.Cancer 1995,61,381-388

(58) Field of Search
UK CL (Edition Q) G1B BAB BAD BBD
INT CL[6] C12Q 1/48 , G01N 33/573 33/574
ONLINE: WPI,CLAIMS,BIOSIS PREVIEWS,CAB Abstracts,EMBASE,ChemEng & Biotec Abs, Derwent Biotechnology Abs,Current BioTech Abs

(54) Abstract Title

Diagnosis of cancer involving assay of levels of cyclin-dependent kinase (CDK) isoenzymes

(57) A process for the diagnosis of a cancerous, or pre-cancerous, state in a subject comprises assay of a sample comprising cells (preferably mutant p53 cells), or an extract therefrom, for the co-elevation of CDK1 and CDK4.

The assay may be performed by contacting the sample with labelled antibody against CDK1 and/or labelled antibody against CDK4.

A kit for performing the diagnosis is described.

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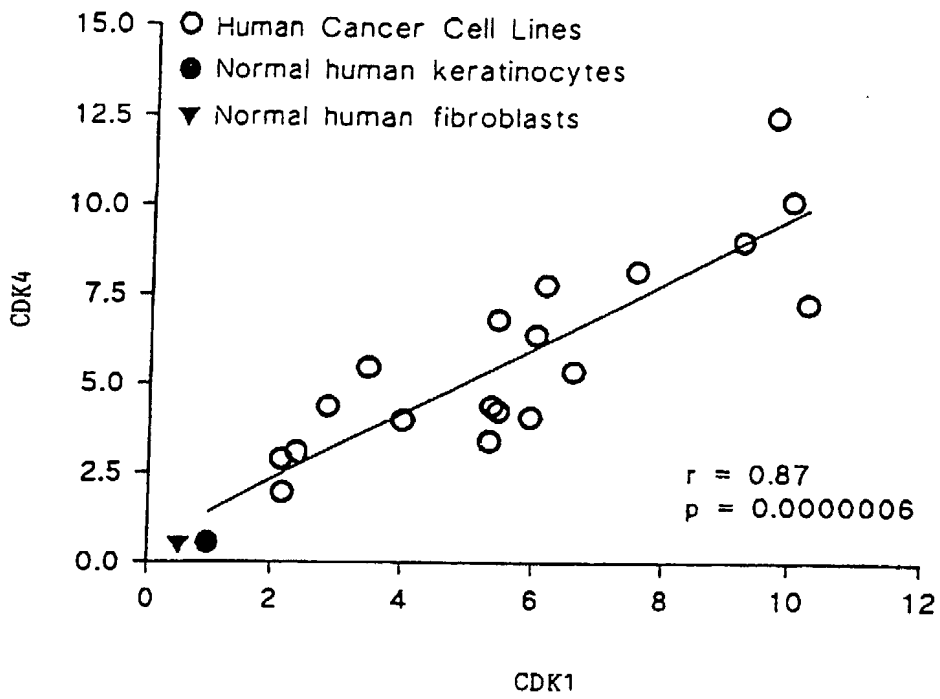
FIGURE 1

A



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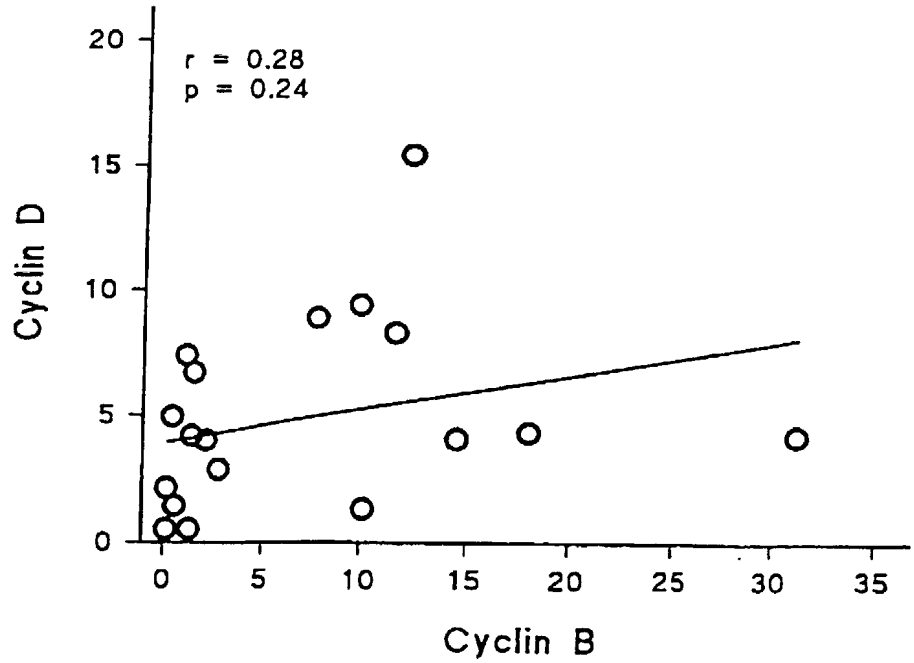
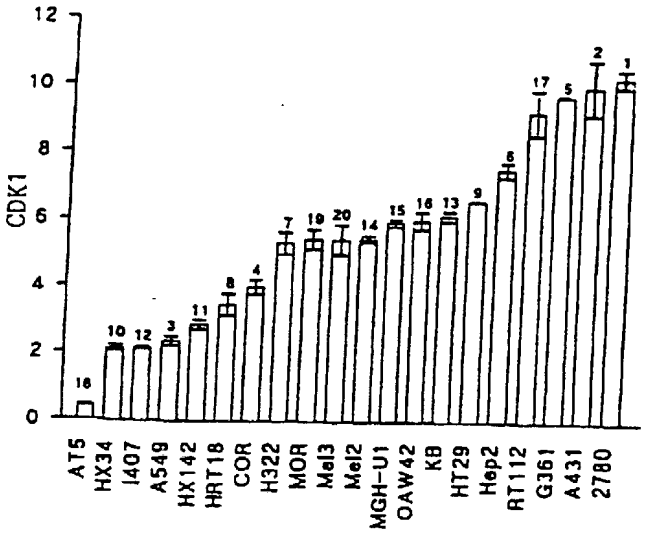


FIGURE 2

A



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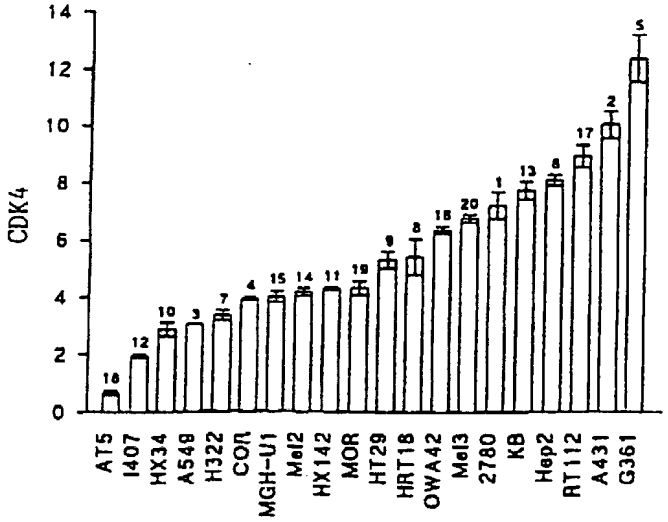


FIGURE 3

A

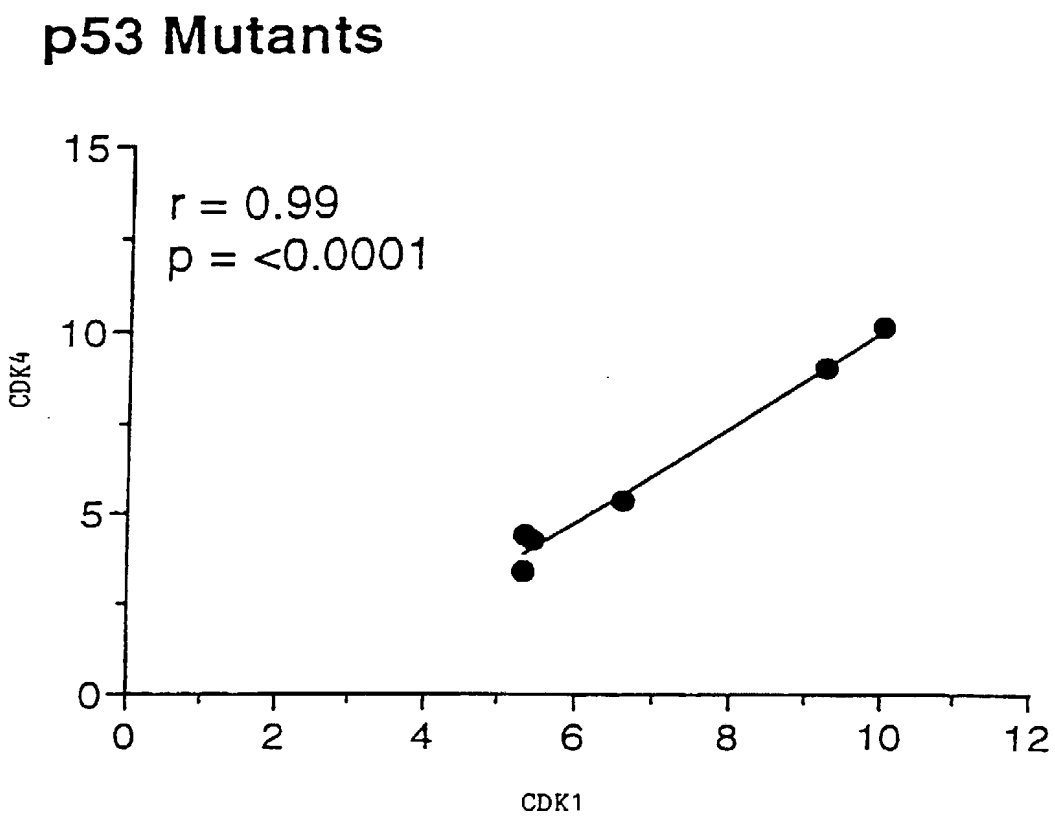
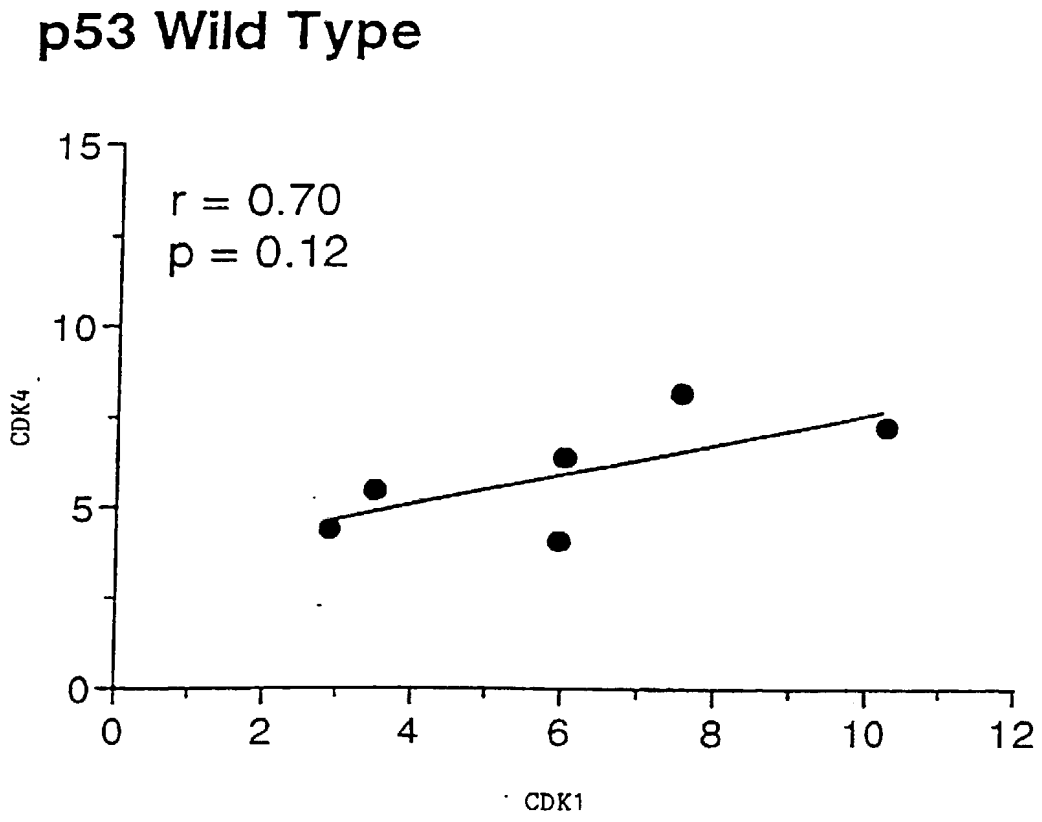


FIGURE 3

B



CANCER DIAGNOSIS

The present application concerns methods of diagnosing cancer. In particular the application concerns a method for diagnosing cancer by measuring the levels of two proteins present in suspected cancer cells.

The hallmark of cancer is a capacity for unlimited, autonomous cellular proliferation. The discovery of a means of selective inhibition of proliferation in cancer cells without concomitant damage to the proliferative capacity of normal cells, could potentially provide new ways to halt the growth of tumours irrespective of their degree of differentiation, invasion or metastasis. We are therefore very interested in understanding how cancer cells divide and whether this may differ from normal cell division. Most treatments for cancer are broadly cytotoxic, generally targetting proliferating cells. Normal tissues also proliferate, however, and so are also damaged by cytoxic agents. Identification of cancer specific markers that permit targetting or that provide specific targets for drug development would allow the development of treatments that are more specifically toxic to tumour tissues thus reducing the debilitating effects of chemotherapy.

Important co-relationships have been identified between certain independently expressed cancer genes providing previously undescribed targets against which to direct therapy that is more specifically toxic to cancer tissues. Cyclin-dependent kinases (CDKs) are genes which are critically important in controlling cell division in normal cells. CDK4 and CDK1 control progress through the G1/S checkpoint and G2/M cell cycle checkpoints respectively.

This application specifically deals with measuring the levels of CDK1 protein and levels of CDK4 protein, in cells. Preferably the p53 mutational status of the cells has been identified by DNA sequencing. High levels of CDK1 and CDK4 are found in human

cancer cells, especially those bearing p53 mutations.

Modulation of the relationship between CDK1 and CDK4 in p53 Mutant Human cells

The above are unusual findings that do not fit with what is widely known about CDKs at the present time. Disruption of the CDK1/CDK4 relationship in p53 mutant cells can be identified as a new target for anticancer therapy. In addition because both the CDK1/CDK4 co-elevation and p53 mutation are confined to cancer cells and appear to be inter-related, they form in combination a complex target that is likely to prove the most specific one for cancer therapy that has so far been discovered.

This invention describes methods of diagnosing a cancerous state by contemporaneously measuring the properties of two or more cancer-related genes. This application specifically deals with measuring the levels of the protein product of the CDK1 gene and the protein product of the CDK4 gene whilst also determining the mutational status of p53.

Specifically this invention provides a method for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising testing a sample comprising cells or an extract therefrom for the co-elevation of the level of CDK1 and the level of CDK4. The invention also provides a kit for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising a means for testing for the elevation of the level of CDK1 and a means for testing for the elevation of the level of CDK4.

Furthermore this invention provides a novel, complex, target for drug screening which might lead to drugs that are more specifically toxic to cancer tissues with the features disclosed in this application.

The invention will now be described in further detail by way of example only, with reference to the accompanying drawings, in

which:-

Figure 1A shows the correlation between CDK1 and CDK4 levels in human cancer cell lines as well as the corresponding levels in "normal" cells such as human keratinocytes and human fibroblasts;

Figure 1B shows the relative lack of correlation between cyclin D and cyclin B (the respective partners of CDK4 and CDK1) levels in human cancer cell lines;

Figure 2A shows the range of values for CDK1 levels found from several Western immunoblot runs carried out on specific human in vitro cell lines (the standard errors are also shown);

Figure 2B shows the corresponding data to Figure 2A, obtained in respect of CDK4;

Figure 3A shows the correlation between CDK1 and CDK4 levels in p53 mutant human cancer cell lines; and

Figure 3B shows the corresponding correlation to Figure 3A, obtained in respect of wild-type p53 human cell lines.

A Dual Parameter Test for Cancer using CDK1 and CDK4 Protein Expression

A clinical test for cancer is proposed based on the measurement of CDK1 protein expression levels, CDK4 protein expression levels and detection of mutations in the p53 gene. In a research environment CDK protein levels are typically measured by Western blotting or by immunocytochemistry but for diagnostic purposes cheaper and more rapid methods are more desirable.

The determination of the mutational status of p53 can be effected by sequencing the genomic locus bearing the gene from the patient or by sequencing the expressed mRNA after conversion to cDNA. Various nucleic acid sequencing methodologies are available at

present, all of which are appropriate for use with this diagnostic assay. The most widely used method would be based on incorporation of terminating nucleotides into polymerase generated copies of a template, using the method of Sanger et al, 1977. Many alternatives have arisen recently including adaptor sequencing (PCT/US95/12678) , ligation based sequencing (PCT/US96/05245), sequencing by hybridisation (A.D. Mirzabekov, TIBTech 12: 27 - 32, 1994) to list a few. Various methods for testing for specific mutations exist and are well known in the art, such as the TaqMan assay, oligonucleotide ligase assays, single strand conformational polymorphisms and assays based on hybridisation of template nucleic acids to oligonucleotide arrays.

Because CDK1 and CDK4 are not under direct transcriptional control, it is unlikely that mRNA levels for CDK1 and CDK4 will follow the same pattern as their proteins. This means that determination of the mRNA levels of these genes is not sufficient for the purposes of this test.

Immunocytochemistry:

In a preferred embodiment of this invention, CDK1 and CDK4 protein levels could be measured by immunocytochemistry using confocal laser fluorescence microscopy to detect antibody binding. Preferrably a scanning system would be used such as those described in PCT/US91/09217, PCT/NL/00081 and PCT/US95/01886. An antibody against CDK1 (such as the Mouse monoclonal sc-54 from Santa Cruz Biotechnology, CA) would be labelled with one dye, an antibody against CDK4 (such as the purified rabbit polyclonal sc-260 from Santa Cruz Biotechnology, CA) would be labelled with a second dye whilst a third DNA binding dye could be used to select for aneuploid cells. DNA binding dyes such as Hoechst 33258 dye, which binds AT-rich DNA or Chromomycin A_3, which binds GC-rich DNA, would be appropriate. The intensity of fluorescence from these dyes would provide an indication of the expression level of the genes. A diagnostic

test might comprise the steps of:

- Extracting a biopsy of the tumour from a patient.
- Optionally micro-dissecting that material to separate normal tissue from tumour material.
- Preparing the biopsy material for microscopy which includes the steps of:
  - Labelling the biopsy material with the above fluorescently labelled antibody probes against CDK1, CDK4. The biopsy material may also, optionally be labelled with antibody probes against p53 mutant proteins and with a DNA binding dye.
  - Separating the labelled cells from unbound labelled probes.
- Placing the labelled biopsy material in a scanning confocal microscope to count cells that:
  - Over-express or show elevation of CDK1, i.e. are labelled with at least a threshold quantity of antibody against CDK1.
  - Over-express or show elevation of CDK4, i.e. are labelled with at least the threshold quantity of antibody against CDK4.
  - Optionally, express mutant forms of p53, i.e. are labelled with at least the threshold quantity of antibodies against p53 mutants. Alternatively, p53 mutational status might be determined by analysis of the mRNA or genomic DNA as discussed above.
  - Optionally, have chromosomal amplifications as detected by the intensity of fluorescence from DNA binding fluorescent dyes.

Fluorescence Activated Cell Sorting:

One embodiment of the diagnostic test could exploit Fluorescence Activated Cell Sorting (FACS). A FACS instrument separates cells in a suspension in a manner dependant on the cells being labelled with a fluorescent marker. A typical FACS device operates as follows. Cells in a suspension travelling in single file are passed through a vibrating nozzle which causes the formation of droplets containing a single cell or none at all. The droplets pass through a laser beam. Fluorescence from each individual cell

in its droplet, excited by a laser, is measured. After the detector the stream of cells in suspension pass through an electrostatic collar which gives the droplets a surface charge. The cell carrying droplets are given a positive or negative charge. If the drop contains a cell that fluoresces with an intensity above a particular threshold, the drop gets a charge of one polarity. Unlabelled cells get a charge of the opposite polarity. The charged droplets are then deflected by an electric field and, depending on their surface charge, are directed into separate containers and are counted. Droplets that contain more than one cell scatter light more than individual cells. This is readily detected and so these are left uncharged and enter a third disposal container.

Multi-channel fluorescent detection devices have been constructed that can separate cells on the basis of labelling with multiple different fluorescent labels. These have multiple lasers which can excite fluorescence at different frequencies and the detector will detect different emission frequencies. Using this technique a test could be carried out using a multi parameter array on a flow cytometer without the need for sorting. A three label system would be appropriate for this test. An antibody against CDK1 would be labelled with one dye, an antibody against CDK4 would be labelled with a second dye whilst a third, DNA binding dye could be used to select for aneuploid cells. DNA binding dyes such as Hoechst 33258 dye, which binds AT-rich DNA, or Chromomycin A_3, which binds GC-rich DNA, would be appropriate. Additionally, a number of antibodies are commercially available which can detect some of the mutant forms of p53. Antibodies such as these might be labelled with a fourth dye. The intensity of fluorescence from these dyes would give an indication of the expression levels of the two proteins and would indicate the chromosomal status of labelled cells passing the detector. A minimum level of fluorescence intensity from each dye present in an individual cell would be required to classify a cell as being cancerous. At present not all mutant forms of p53 can be detected using antibodies, although antibodies exist against a number of

known mutant forms of the p53 protein. A diagnostic test might comprise the steps of:

- Extracting a biopsy of the tumour from a patient.
- Optionally micro-dissecting that material to separate normal tissue from tumour material.
- Disrupting intracellular adhesion to form a single cell suspension.
- Labelling the suspended cells with the above fluorescently labelled probes against CDK1, CDK4. The biopsy material may also, optionally be labelled with antibody probes against p53 mutant proteins and with a DNA binding dye.
- Separating the labelled cells from unbound labelled probes.
- Passing the labelled cell suspension through a FACS device to count cells that:
  - Over-express or show elevation of CDK1, i.e. are labelled with at least a threshold quantity of antibody against CDK1.
  - Over-express or show elevation of CDK4, i.e. are labelled with at least the threshold quantity of antibody against CDK4.
  - Optionally, express mutant forms of p53, i.e. are labelled with at least the threshold quantity of antibodies against p53 mutants. Alternatively, p53 mutational status might be determined by analysis of the mRNA or genomic DNA as discussed above.
  - Optionally, have chromosomal amplifications as detected by the intensity of fluorescence from DNA binding fluorescent dyes.

Example:

Human in-vitro cell lines of different histological origin which exhibit a range of intrinsic sensitivity to cytotoxic drugs as measured by clonogenic cell survival assays, have been shown to provide appropriate models of clinical tumours, particularly in their responses to chemotherapy. In particular, these cell lines exhibit the range of sensitivities to cytotoxic drugs and ionising radiation usually encountered in the clinic. These human in-vitro cancer cell lines are now widely recognised as relevant

models for the clinical response of tumours to chemotherapy. It is therefore possible to identify genes from these cancer models whose expression and/or mutational status is tumour specific, which are also features of real clinical tumours. Discoveries in human in vitro cell lines, such as those leading to this invention, therefore, have a strong possibility of being able to be translated into meaningful targets for drug discovery programs.

A body of work has been carried out analysing the expression of a number of genes that have been implicated in the cancer disease process. In contrast to any of the other genes controlling cell division that were examined, CDK1 and CDK4 appeared to be consistently co-elevated in a large series of human cancer cell lines and extracts from clinical colon cancer. It is hypothesised that for cancer cells to be able to continue to divide successfully it may be necessary a) for CDK1 and CDK4 to retain their normal functions and b) for the elevated levels of these two proteins in human cancer to be related in some way, the mechanism of which was unclear.

To test the above hypothesis DNA sequencing has been carried out on all the exons of both CDK1 and CDK4 in the 20 human cell lines in which a strong relationship between the expression of the two proteins had been detected. There were no mutations in the exons of the CDK1 and CDK4 genes in the 20 human in-vitro cell lines that were sequenced. This finding is very surprising, because cancer cells are well known to progressively accumulate mutations in the critical genes controlling cell division and this is held by many scientific practitioners in this field to be most marked in human in-vitro cell lines such as those investigated.

Materials and Methods:

<u>Cell lines and clonogenic cell survival assays</u>

The growth characteristics clonogenic assay procedures of the 14

human in vitro cell lines used in this analysis have already been reported (Warenius et al 1994). The cell lines are listed, with their histological classification in Table 1. All are well established; many having been growing in vitro for several years. Cell lines were either donations or purchased by our laboratories. On receipt all were grown for 5 passages to provide sufficient cells for batch storage in liquid nitrogen. During this period contamination was excluded by at least one passage in antibiotic free medium and mycoplasma testing was carried out on all lines. For clonogenic assays, cells were taken from a designated primary liquid nitrogen batch and grown for 3-6 passages until there were sufficient well-growing cells. Further batches from these cells were frozen in liquid nitrogen. Cells were routinely maintained in DMEM medium except RT112 and H322, which were grown in RPMI1640 and MGHU-1 which were grown in Ham's F12 medium. All lines were supplemented with 10% heat-inactivated fetal calf serum (HIFCS).

Table I. p53 mutational status

	Cell Line	cDNA sequence	Amino-acid change	p53 protein
I407	Embryonic intest. epith.	Normal	none	Wild-type
HEP 2	Squamous carcin. larynx	Normal	none	Wild-type
MGHU 1	Transit. carcinoma bladder	Normal	none	Wild-type
HRT 18	Adenocarcinoma rectum	Normal	none	Wild-type
2780	Ovarian carcinoma	Normal	none	Wild-type
OAW 42	Ovarian carcinoma	CGA-CGG codon 213	none	Wild-type
HT 29/5	Adenocarcinoma colon	CGT-CAT codon 273	Arg-His	Mutant
COLO 320	Adenocarcinoma colon	CGG-TGG codon 245	Arg-Tryp	Mutant
H 322	Small cell carcinoma lung	CGG-TGG codon 245	Arg-Tryp	Mutant
H 417	Small cell carcinoma lung	GAG-TAG codon 298	Glu-Stop	Truncated
RPMI 7951	Melanoma	TCA-TTA codon 166	Ser-Stop	Truncated
RT 112	Transit. carcinoma bladder	CCG-CAG codon 248	Arg-Gly	Mutant
MOR	Adenocarcinoma lung	C deletion codon 152	Frameshift 178αα	Truncated
MEL 2	Melanoma	CGC-AGC codon 245	Gly-Ser	Mutant

Identification of mutations in the p53 gene by PCR and DNA sequencing.

Material for PCR and DNA sequencing of p53 and Western blotting for Raf-1 protein, was obtained from the same liquid nitrogen batches used to provide cells for clonogenic cell survival data. Cells were grown for up to three passages prior to being subjected to the following procedures:

Nucleic Acid Isolation

RNA and genomic DNA were prepared from the cell lines described here by the guanidinium isothiocyanate CsCl gradient method (Chirgwin et al, 1979, Barraclough et al, 1987). Briefly, the cells were collected in ice-cold phosphate-buffered saline (PBS) and homogenised in guanidinium isothiocyanate buffer (4M guanidinium isothiocyanate, 50mM Tris pH 7.5, 25mM EDTA pH 8.0, 0.5% (w/v) sodium lauryl sarcosine and 8% (v/v) 2-mercaptoethanol added just prior to use. The homogenate was cleared by centrifugation at 8,000 rpm for 10 mins at 4°C (SS34 rotor, Sorvall RC-5B centrifuge) and the RNA pelleted by centrifugation of the homogenate through a cushion of 5.7M caesium chloride/0.1M EDTA at 32,000 rpm for 20hr at 20°C (TST 41.14 rotor, Kontron Centrikon T20 60 ultracentrifuge). The pellet of RNA was redissolved in 0.1% (w/v) SDS and precipitated with ethanol overnight at -20°C before quantitation.

Polymerase Chain Reaction, cDNA synthesis and nucleotide sequencing

PCR (for exons 2-8 and for exons 9-11) was performed on DNA and RNA extracted from 18 human carcinoma cell lines. Each exon was then examined by DNA sequencing. PCR Primers were designed flanking each exon and synthesised on an Applied Biosystems 381A DNA synthesiser. Each exon was amplified separately with the exceptions of exons 2 and 3 which were amplified as a unit, and exons 9, 10 and 11 which were amplified together by reverse

transcription polymerase chain reaction (RTPCR). The following primers were used:-

Exon 2/3 sense 5'-CCC ACT TTT CCT CTT GCA GC-3'

Exon 2/3 antisense 5'-AGC CCA ACC CTT GTC CTT AC-3'

Exon 4 sense 5'-CTG CTC TTT TCA CCC ATC TA-3'

Exon 4 antisense 5'-GCA TTG AAG TCT CAT GGA AG-3'

Exon 5 sense 5'-TGT TCA CTT GTG CCC TGA CT-3'

Exon 5 antisense 5'-CAG CCC TGT CGT CTC TCC AG-3'

Exon 6 sense 5'-GCC TCT GAT TCC TCA CTG AT-3'

Exon 6 antisense 5'-TTA ACC CCT CCT CCC AGA GA-3'

Exon 7 sense 5'-ACT GGC CTC ATC TTG GGC CT-3'

Exon 7 antisense 5'-TGT GCA GGG TGG CAA GTG GC-3'

Exon 8 sense 5'-T ATC CTG AGT AGT GG-3'

Exon 8 antisense 5'-T GCT TGC TTA CCT CG-3'

Exon 9/10/11 sense 5'-AGA AAG GGG AGC CTC ACC AC-3'

Exon 9/10/11 antisense 5'-CTG ACG CAC ACC TAT TGC AA-3'

Genomic DNA was digested with EcoR1 and precipitated with ethanol and resuspended in 50μl of water (Sigma) before being subjected to PCR amplification. The DNA (1μg) was amplified in 50μl PCR reactions containing 20 pmoles of each primer. A 'hot start' PCR protocol was used with the dNTP's and Taq enzyme initially separated from the rest of the reaction components on a wax

cushion. The reactions were placed in a pre-heated PCR block at 95°C for 2 minutes before undergoing thirty cycles of denaturation (30s at 95°C), annealing (30s at 60°C for exons 2-3, 4 and 6; 65°C for exons 5 and 8; 67°C for exon 7; and 68°C for exon 9-11) and extension (1 min at 72°C). The PCR products were checked on a 0.8% (w/v) agarose gel before being purified using a Wizard minicolumn (Promega), and used directly in sequencing reactions.

cDNA synthesis and RTPCR

Complementary DNA was synthesised from approximately 5μg of total RNA using oligo (dT) as a primer. Total RNA (5μg), human placental ribonuclease inhibitor (HPRI) 20U and 1μg oligo (dT) were heated at 70°C for 10 minutes, chilled on ice, added to 1x first strand buffer (50mM Tris-HCl, pH 8.3, 75mM potassium chloride and 3mM magnesium chloride), 0.01M DTT, dNTPs (0.5mM for each deoxyribonucleoside triphosphate), 400U of Superscript Reverse Transcriptase (Gibco) and incubated at 37°C for 1 hour. PCR for exons 9 to 11 was carried out using 5μl of the above incubation in a 50μl of PCR reaction as described in the previous section.

Nucleotide Sequencing

Sequencing primers (10 pmoles) were radioactively labelled at their 5' ends with γ^{32}P-ATP (45μCi) at 37°C for 30 min in a reaction containing T4 Polynucleotide Kinase (PNK) (9.7U, Pharmacia) and 1x T4 PNK buffer (10mM Tris-acetate, 10mM magnesium acetate and 50mM potassium acetate). The primers used were identical to those employed in the PCR reactions except for exon 5 for which a separate sense sequencing primer was designed as follows:- 5'-TAC TCC CCT GCC CTC-3'. Sequencing was carried out by the dideoxynucleotide enzymatic method (Sanger et al, 1977), using the fmol DNA Sequencing System (Promega). Any putative sequence mutations identified were confirmed by additional sequencing of the exon in the antisense direction as

well as by carrying out a repeat PCR and sequencing of the cell line.

Western Blotting for CDK1 and CDK4.

Two independent Western blottings with lysates for each cell line loaded in pairs on each gel were carried out. 10^7 cells were grown in 162 cm^2 tissue culture flasks (Costar Ltd., High Wycombe, Bucks until they were pre-confluent but still growing exponentially. Cells were then removed by trypsinisation, resuspended in complete medium + 10% FCS and washed 3 times by serial centrifugation and resuspension in PBS without serum. 1-3 x 10^8 viable cells were then pelletted by centrifugation and resuspended at $3x10^7$ cells per ml of lysate buffer (Stock solution: 10% SDS 10ml., 0.5M Tris pH 6.8, glycerol 10 ml., Double distilled water 62 ml. To 10 ml. of stock solution were added 100 ml of 10 mM Leupeptin + 10 ml 100 mM PMSF). Protein estimations were performed and the final concentration of the lysates adjusted to 300 μg total cellular protein per 100 μl. To measure CDK1 and CDK4 proteins, 150μg of total cellular protein in 50 μl of lysate buffer was added per lane well to a 7.5% Laemmli separating gel and electrophoresis carried out at 16°C using 60V over 16 hours and a constant current of 500mA. Blots were transferred to nitrocellulose at 22°C over 16 hours using to a semi-dry blotting apparatus (Biorad, Richmond, CA). To determine CDK1 protein expression levels the blot was incubated with the sc-054 mouse monoclonal antibody to human CDK1 (Santa Cruz Biotechnology, CA) and then incubated with rabbit anti-mouse conjugated antibodies (Dako UK) at 1/1000 and developed in alkaline phophatase buffer containing Nitroblue Tetrazolium and 5-Bromo-4-Chloro-3-Indoyl Phosphate, (Sigma, Poole, Dorset, UK) (50mg/ml in dimethylformamide). To determine CDK4 protein expression levels the blot was incubated with the sc-260 rabbit poly-clonal antibody to human CDK4 (Santa Cruz Biotechnology, CA) and then incubated with rabbit anti-mouse conjugated antibodies (Dako UK) at 1/1000 and developed in alkaline phophatase buffer containing Nitroblue Tetrazolium and 5-Bromo-4-Chloro-3-Indoyl

Phosphate, (Sigma, Poole, Dorset, UK) (50mg/ml in dimethylformamide). Quantitation of the protein product of the CDK1 and CDK4 genes was carried out by measurement of optical density on a Schimadzu scanning densitometer with tungsten light and expressed as O.D. units per 150 μg of total cellular protein. Titration curves obtained by loading different amounts of total cellular protein have previously shown that linear relationships for optical density (O.D.) could be obtained over the range found for CDK1 and CDK4 protein across the cell lines. In order to compare different CDK1 and CDK4 protein levels between the cell lines, the mean O.D. value for all the lines was calculated and the relative O.D. for CDK1 and CDK4 protein in each individual cell line was normalised to the mean O.D. and multiplied by an arbitrary value of 5.0.

The cell lines investigated are shown in Table 2.

TABLE 2

Cell Line	Histology
2780	Ovarian carcinoma
A431	Squamous carcinoma vulva
A549	Adenocarcinoma lung
AT5BIVA	Ataxia telangiectasia transformed fibroblast
COR L23	Large cell lung carcinoma
G361	Melanoma
H322	Small cell carcinoma lung
HEP2	Squamous carcinoma larynx
HRT18	Adenocarcinoma rectum
HT29/5	Adenocarcinoma colon
HX142	Neuroblastoma
HX34	Skin melanoma
I407	Embryonic intestinal epithelium
KB	Oral epidermoid carcinoma
MEL2	Melanoma
MEL3	Melanoma
MGHU-1	Transit cell carcinoma bladder
MOR	Adenocarcinoma lung
OAW42	Ovarian carcinoma
RT112	Transit cell carcinoma bladder

The results are shown in Figures 2A and 2B.

Conclusions:

A strong correlation between the expression of the CDK1 and CDK4 in a series of 20 human in-vitro cancer cell lines is observed. Using DNA sequencing data for p53 mutational status for a number of these lines, it is further observed that the CDK1/CDK4 relationship is particularly marked in cell lines with p53 mutations and the correlation is very strong indeed in p53 mutant cell lines.

Also surprisingly the correlation seen between the protein expression for these cell lines was not seen for the relevant mRNAs. Nor was there a reciprocal correlation between the protein expression for one gene and the mRNA for the other. These results suggest that the co-elevation of CDK2 and CDK4 proteins is not at the level of transcriptional control but may be at a transalational or post-transalational stage. That is to say the proteins themselves are influencing each other in some way or are both influenced by an as yet unknown factor.

The findings concerning the co-relationships of CDK1 and CDK4 proteins in human in-vitro cell lines and clinical colon cancers are supported by transfection studies into RAMA37, a rat normal myoepithelial cell line. Pilot studies with RAMA37 suggest that overexpression of CDK4 protein as a result of successful transfection of CDK4 under unconditional promoter control is accompanied by a concomitant elevation in the constitutive expression of CDK1.

References:

Barraclough et al, J. Cell Physiolog **131**: 393 - 401, 1987.

Chirgwin et al, Biochemistry **18**: 5294 - 5299, 1979.

Sanger et al, Proc. Natl. Acad. Sci. USA **74**: 5463 - 5467, 1977.

Warenius et al., Int.J.Cancer. **67**: 224 - 231, 1996.

CLAIMS:

1. A method for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising testing a sample comprising cells or an extract therefrom for the co-elevation of CDK1 levels and CDK4 levels.

2. A method according to claim 1, wherein the sample is extracted from the subject.

3. A method according to claim 1 or claim 2, wherein the testing comprises contacting the sample with a labelled antibody against CDK1 and/or a labelled antibody against CDK4.

4. A method according to claim 3, wherein the antibody against CDK1 is sc-54 (Santa Cruz Biotechnology Inc., CA) and/or the antibody against CDK4 is sc-260 (Santa Cruz Biotechnology Inc., CA)

5. A method according to any preceding claim, wherein the cells are mutant p53 cells.

6. A method according to claim 5, wherein mutant p53 cells are identified by contacting the sample with a labelled antibody against mutant p53.

7. A method according to any of claims 3-6, wherein at least one antibody is labelled with a fluorescent label.

8. A method according to any preceding claim, wherein the testing is carried out using Western blotting.

9. A method according to any preceding claim, wherein the sample is a sample of cells.

10. A method according to claim 9, wherein the testing is carried out by performing a cell count.

11. A method according to claim 10, wherein the cell count is performed using multi-parameter flow cytometry.

12. A method according to claim 10, wherein the cell count is performed using scanning confocal microscopy.

13. A method according to claim 10, wherein the cell count is performed using fluorescence activated cell sorting.

14. A method according to any of claims 10-13, wherein the sample of cells is micro-dissected prior to performing the cell count, to separate normal tissue from tumour tissue.

15. A method according to claim 14, wherein normal tissue is separated from tumour tissue by contacting the sample of cells with a DNA binding dye to label aneuploid cells, and separating labelled cells from non-labelled cells.

16. A method according to claim 15, wherein the DNA binding dye is Hoechst 33258 or Chromomycin A_3 dye.

17. A method according to any of claims 10-16, wherein prior to performing the cell count, intracellular adhesion in the sample of cells is disrupted, to form a single cell suspension.

18. A method according to any preceding claim, wherein the CDK1 and CDK4 are wild-type CDK1 and CDK4.

19. A kit for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising a means for testing for the elevation of CDK1 levels and a means for testing for the elevation of CDK4 levels.

20. A kit according to claim 19, wherein the means for testing for the elevation of CDK1 levels comprises a labelled antibody against CDK1 and the means for testing for the elevation of CDK4 levels comprises a labelled antibody against CDK4.

21. A kit according to claim 20, wherein the antibody against CDK1 is sc-54 (Santa Cruz Biotechnology Inc., CA) and the antibody against CDK4 is sc-260 (Santa Cruz Biotechnology Inc., CA).

22. A kit according to any of claims 19-21, further comprising a means for identifying mutant p53 cells.

23. A kit according to claim 22, wherein the means for identifying mutant p53 cells comprises a labelled antibody against mutant p53.

24. A kit according to any of claims 20-23, wherein at least one antibody is labelled with a fluorescent label.

25. A kit according to any of claims 19-24, further comprising a DNA binding dye, for labelling aneuploid cells.

26. A kit according to claim 25, wherein the DNA binding dye is Hoechst 33258 or Chromomycin A_3 dye.

27. A kit according to any of claims 19-26, wherein the CDK1 and CDK4 are wild-type CDK1 and CDK4.

The Patent Office

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Application No: GB 9803447.3 **Examiner:** Colin Sherrington
Claims searched: 1 to 27 **Date of search:** 21 January 1999

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.Q): G1B(BAB,BAD,BBD)

Int Cl (Ed.6): C12Q 1/48; G01N 33/573,33/574

Other: ONLINE: WPI, CLAIMS, BIOSIS PREVIEWS, CAB Abstracts, EMBASE, ChemEng & Biotec Abs, Derwent Biotechnology Abs, Current BioTech Abs

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	WO 97/04316 A1 (PARACELSIAN, INC.) -whole document, especially page 9, lines 19 to 27; EXAMPLES 10 to 18; claims 1, 4	1
A	Medecine Sciences 1996,12(2),165-173 -Dariusz Wolowiec *et al.* "Kinases dependantes des cyclines: role biologique et implications dans la pathologie humaine"	
A	Leukemia 1995,9,1382-1388 -D.Wolowiec *et al.* "Expression of cell cycle regulatory proteins in chronic lymphocytic leukemias. Comparison with non-Hodgkin's lymphoma and non-neoplastic lymphoid tissue"	
A	Int.J.Cancer 1995,61,381-388 -Dariusz Wolowiec *et al.* "CDK1 is a Marker of Proliferation in Human Lymphoid Cells"	

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